Studies in the Experimental Analysis of Sex.

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Part 7.—Sexual Changes in the Blood and Liver of Carcinus mænas.

In the preceding parts of these studies evidence has been brought forward to show that the adult female organism at the time of the ripening of the ovary is engaged in elaborating reserve material, especially of a fatty nature, that this substance is conveyed in the body-fluids to the ovary, and we have seen reason for associating the presence of this substance in the circulation with the development of many of the female secondary sexual characters. It was especially in connection with the effect of the parasite Sacculina upon its host Inachus that the probable importance of this process was recognised, and the hypothesis was advanced that the underlying cause for the assumption of adult female characteristics by both sexes of infected crabs was the fact that the Sacculina forced them to elaborate a fatty or yolk material similar to that which the normal female crab produces at the time that the ovary is ripening. Attention was called to the fact that the roots of the Sacculina actually do elaborate a yolk material from the blood of the host, similar in appearance and in staining reactions to the yolk which the adult female normally stores in its ovary.

At the time when these observations were put forward, I did not suspect that a means was ready to hand for testing this hypothesis, and that the French observer Heim (1) several years ago had observed that the blood of many female crabs at the time of the ripening of the ovary becomes charged with a brilliantly coloured yellow or orange lipochrome, which, according to Heim, is entirely absent from the male. Since lipochrome is invariably associated with fatty substances, in which it is soluble, it was at once apparent that Heim's observation had a very direct bearing upon our problem; at my suggestion, therefore, Mr. G. C. Robson undertook to investigate the question in Inachus at Naples, while parallel observations have been made by myself on Carcinus mænas at Plymouth.

As far as the course of events in normal uninfected crabs is concerned, Robson's observations on Inachus and my own on Carcinus agree in every particular, but in the crabs infected with Sacculina the two sets of material yield different results. As will be seen in the course of this paper, this difference may be ascribed to the fact that the general effect of Sacculina on Carcinus is far less marked than it is on Inachus, so that we might expect the same to be true of the effect of the parasite upon the blood-properties of its hosts.

The blood of Carcinus exhibits three chief types of variation with respect to colour; it may be pale blue, owing to the presence of hæmocyanin, in which case it may be spoken of as non-pigmented or colourless; it may be of a salmon-pink colour owing to the presence of the lipochrome, tetronerythine, which masks the blue colour of the hæmocyanin, or it may be yellow owing to the presence of a different lipochrome, which may be called lutein. The presence or absence of these substances in the blood follows certain definite phases of the animal's existence. The blood is invariably colourless or practically free from lipochrome immediately at and after a moult, and also in the case of the female after the eggs have been shed to the exterior. It

may remain colourless for some time after these processes, but gradually as the crab approaches the period of a new moult or of the formation of a fresh batch of eggs, lipochrome accumulates in the blood in a manner characteristic for each sex. In the male sex the pink lipochrome invariably preponderates, so that the blood of the male is characteristically of a salmon-pink colour; in the female sex, on the other hand, the yellow lipochrome preponderates very strongly over the pink, and this is most markedly the case when the female is ripening the ovary and storing up the lutein in it. Often intermediate orange colours appear in both sexes, due to an intermixture of the two pigments, but in the vast majority of cases the blood of the male is either colourless or pink, that of the female colourless or yellow. That the appearance of the brilliant yellow colour in the blood of the female is strictly associated with the ripening of the ovary has been completely settled by Robson and myself, and in this respect we agree with Heim's observations on a number of other Decapod Crustacea, but the occurrence of the pink lipochrome in the male sex at the periods leading up to the moult is no less certain, though Heim appears to have missed it. And it must be admitted that at first sight the occurrence of the lipochrome in the normal male, which, as far as intensity of colour is concerned, appears to be present in quantities equal to that in the female, offers a serious difficulty to our hypothesis. For it would seem to indicate that periodically the male blood is charged with fatty material to the same extent as the female.

An unexpected light has been thrown upon this difficulty by a series of quantitative estimations of the fat-content of the blood of *Carcinus mænas* which I have recently made. The method which I have used in making these estimations is one which is recommended by Professor Leathes in his book on the fats (**2**), and my exact mode of procedure is as follows: About 20 gr. of blood is drawn from a number of crabs and accurately weighed on a chemical balance into a porcelain dish. An equal weight of a 1·30 per cent. solution

of caustic potash is added and the mixture is heated on a water-bath for about half an hour. About 10 c.c. of alcohol are added and the heating is continued for another hour. By this process the proteid material of the blood is largely broken down and all the fat and lipoid substances are converted into soap. The saponified mixture is now washed with hot water into a long-necked flask, and a sufficient quantity of 40 per cent. sulphuric acid is carefully added until the reaction is distinctly acid. By this means the fatty acids are liberated from the soaps. After cooling, 40 c.c. of pure ether petroleum are added, and the flask, after being stoppered, is shaken for about an hour. The fatty acids are taken up by the petroleum, and water is added to the flask until a clear column of petroleum is obtained in the neck of the flask. Ten or 20 c.c. of the petroleum is accurately pipetted off and evaporated to dryness in a weighed beaker. The weight of the residue in the beaker gives the amount of ether-soluble substances contained in the blood, after saponification and liberation of the fatty acids, and may be taken as giving a fairly accurate estimate of the amount of fat and lipoid in the original blood. There are certain experimental sources of error in this method, but by always taking nearly the same amount of blood and treating it in the same way, the comparative estimate afforded for different types of blood is reliable and has given very constant results, as the subjoined table shows.

The ethereal extract on evaporating to dryness yielded a golden yellow or orange waxy substance, soluble in alcohol or ether, as a clear yellow or orange solution. The chemical nature of the fat and lipoid substances contained in this material will be the subject of future investigation.

The following points, however, which were made out with the assistance of Dr. Ramsden, are sufficient to indicate the nature of the substances dealt with. A weak ethereal solution of the extract from the pink blood gives when examined spectroscopically an absorption band on the F line of the spectrum, while the yellow lipochrome in solution gives in a suitable dilution two bands, one at the F line and another

at the G. If the solutions of either yellow or pink lipochrome are too strong, a broad diffuse band is formed cutting off the whole of the blue part of the spectrum. These absorption bands are the same as those found by Haliburton and other observers for the lutein of the hen's egg-yolk in the case of the yellow substance, and of the tetronerythrin from the blood and eggs of Crustacea in the case of the red. The waxy mixture of fatty acids and lipochrome, in which a certain amount of cholesterol may be present, contains a mixture of fatty acids, part of which is liquid at room temperature, while the melting-point of another portion is rather above 40° C. The mixture as a whole liquefies between 30° and 40° C.

The blood for the estimations was collected and pooled under three categories, non-pigmented of both sexes, pink from males and yellow from females, and the amount of ether-soluble substances present was calculated as a percentage on 100 gr. of the original blood. The following table gives the results of the experiments.

Percentage of ether-soluble substances in the blood of Carcinus mænas.

Non-pigmented.	Pink.	Yellow.
·073 per cent.	·099 per cent.	·158 per cent.
·087 ,,	·084 ,,	·103 ,,
·035 ,,	·059 ,,	·156 ,,
·074 ,,	·088 ,,	·203 ,,
·034 ,,	·07 ,,	·373 ,,
·086 ,,	·093 ,,	·135 ,,
·058 ,,	·095 ,,	·320 ,,
·045 ,,	·103 ,,	·143 ,,
·035 ,,		
·07 ,,		
·062 ,,		
Average ·059 per cent.	Average ·086 per cent.	Average ·198 per cent.

This table brings out unmistakably the remarkable result that the blood containing the yellow lipochrome not only yields on the average nearly four times as much fatty material as the colourless blood, but also more than twice as much as the blood containing the pink lipochrome.

This result was wholly unexpected, as the pink blood used for the estimation was deeper in colour than the yellow, owing to the fact that during September when the estimations were made, the females were only beginning to ripen their ovaries, so that the blood was generally of only a pale yellow.

There can be no doubt that if a large quantity of deep yellow blood were subjected to the analysis, a still higher percentage, up to ·5 per cent., would be readily obtained. But the results as they stand indicate the remarkable fact that the pink lipochrome stands for a lower fat-equivalent than the yellow. We are therefore in a position to say that the blood of the female at the time of the ripening of the ovary contains at any rate more than twice as much fatty material as the blood of the male, even when the latter is mobilising its fat to the greatest extent.

We have stated that the pink lipochrome is characteristic of the blood of the male, while the yellow is characteristic of that of the female. This is roughly true if we confine ourselves to mere inspection, though an intermediate orange tint is met with in both males and females. The saponification and extraction method which we have explained throws a deeper light into the question of the degree of admixture of the two lipochromes. It is found that the ether petroleum which has been shaken up with the treated blood of any of the three kinds, i.e. non-pigmented, pink or yellow, on evaporation invariably shows the presence of the golden yellow lipochrome, in very small quantities in the non-pigmented blood, in rather greater quantity in the pink blood, and in much greater quantity in the yellow blood. From the pink blood a relatively small amount of pink lipochrome appears in the

extract. It is clear, therefore, that the pink lipochrome represents a very small proportion by weight of the fatty material extracted from the blood, and that for its weight it has a disproportionately great power of colouring the blood.

It is also interesting to observe that the presence in small quantities of the yellow lipochrome can always be detected in the extract obtained from what was apparently quite colourless blood. It is, in fact, impossible, as far as my experience goes, to obtain blood, however colourless, that does not yield a small quantity of the yellow lipochrome. This substance is therefore always present in the blood of both males and females, though the amount of it varies so greatly at different phases of the crab's life, and is enormously increased in the female at the time of the ripening of the ovary.

The difference that we have established between the two sexes in the properties of their blood is therefore a quantitative one, and though qualitative differences may lie behind we have no evidence as yet for their existence. It will, however, be perceived that the results of these estimations have resolved our difficulty with regard to the presence of the lipochrome in the male's blood, since we have shown that the presence of this pink lipochrome in the male indicates a lower fat content than the yellow lipochrome of the female.

Some observations may be conveniently made here as to the fate of these lipochromes and their associated fatty materials, and their probable function in the organism. In the case of the female we know that the yellow lipochrome is taken up by the ovary, which progressively becomes bright yellow as it reaches maturity, while the lipochrome coincidently disappears from the blood. A certain amount of the pink lipochrome also finds its way into the ovary, but it represents an unimportant amount of the reserve material as compared with the yellow. But there is another destination for the pigments, the sole destination for them in the case of the male, and that is the integument. As the males and females pass from the soft green-coloured condition

immediately after a moult and approach the period when a new ecdysis may be expected, the chitinous integument changes from green to yellowish brown or red, especially on the under surface and in the region of the joints. In the male these changes are particularly striking, especially in large males, where the skin at the joints of the appendages becomes bright red. There can be no doubt from the sequence of events that this red pigment which appears between the moults at the joints of the male's limbs is derived from the pink lipochrome in the blood. As to the function of the red deposit, it is difficult to speculate; it may be merely in the nature of an excretion to be got rid of at the ensuing moult, but it is quite possible that it serves as a sexual ornament, since the brilliant red colour of the under-side of the male is fully displayed when he rears himself in combat with other males or in approaching an individual of the opposite sex. The appearance of the lipochrome in the male's blood is, however, an index of a greater fat transport, and it is probable that at the period preceding a moult reserve material is in requisition for the formation of the new skin underneath. We can understand to some extent in this case why the lipochrome, passively accompanying the fat, should appear as a deposit at the surface of the animal's body.

By following the changes in the blood as the various phases of moulting and reproduction follow one another throughout the year it has been possible to establish the conclusions given above. We have now to take a similar survey of the changes occurring in the so-called liver, the great metabolic organ of the Crustacean.

There can be no doubt that Heim was correct in supposing that the liver was the seat of origin of the lipochrome.

Large quantities of fat and lipochrome can always be abstracted from the liver, and sections of the liver stained with osmic acid or Sudan III reveal that the greater number of the liver cells are more or less crowded with fat-globules, which form by far the most important part of the reserve

material in this organ. Now just as the blood varies at different periods in respect of its fat contents, so the liver shows marked variation. Sometimes the liver is opaque white or yellow, and this is a sign that a large quantity of fat is stored up in the fat-cells. Extraction and estimation of the quantity of ether-soluble material, after saponification and treatment by the method given above for the blood, has shown that the liver under these circumstances contains about 12 per cent. of its total weight of fatty material.

Sometimes, however, the liver is seen to be brown in colour and transparent; in this condition the fat-cells are comparatively poorly supplied with fat, and certain other cells, the ferment-cells, are more conspicuous. Analyses of the liver under these conditions show that the fat contents has sunk to 6 or even 4 per cent. of the total weight. This state of fat-exhaustion can be artificially produced by prolonged starvation. Fasting for eleven days gave a fat estimation of 7·38 per cent., for seventeen days 4·46 per cent.

Now observation has shown that when the liver is comparatively free from fat the blood is always colourless, and conversely when the blood is charged with lipochrome a large quantity of fat and lipochrome is present in the liver. It is not, however, the case that the presence of abundant fat in the liver is always accompanied by the presence of fat and lipochrome in the blood, and this is in accordance with expectation on the supposition that the fat and lipochrome is first formed and stored in the liver and then as occasion requires transferred by the blood to the skin or ovary as the case may be. It has been observed that the liver of the female at the time of the ripening of the ovary is always rich in fat and lipochrome, so that we can hardly avoid the conclusion, so plausible on general grounds, that the seat of origin of the fat and lipochrome in the blood is the liver.

We now pass on to consider the effect which the parasite Sacculina exerts upon the properties of the blood and liver which we have described above. Robson and myself agree

that the presence of Sacculina profoundly influences these properties, but the effect is different in the case of Inachus and Carcinus, and presents features difficult to interpret in both.

Robson has made the remarkable discovery that in Inachus the infected individuals of both sexes show the presence of lipochrome in the blood with about the same frequency as normal females that are maturing their eggs, i.e. in respect of the proportion of individuals showing lipochrome in the blood the infected animals of both sexes are in a category which agrees most closely with the breeding females. Now this observation appears to be a complete vindication of the theory which I put forward last year, that the infected individuals would be found to be elaborating a yolk substance similar to that which the female deposits in its ovary at maturity. But there is a puzzling feature in Robson's discovery, and this is that the lipochrome present in the infected crabs is always of the pink tint that is usually associated with the normal individuals soon about to moult, and not of the yellow colour characteristic of the female. It must be admitted that this fact is both unexpected and awkward; it may, however, admit of the following interpretation. The presence of the pink lipochrome at any rate indicates a higher percentage of fatty material in the blood, and this is in support of the theory; the pink lipochrome is very probably only masking a considerable amount of the yellow lipochrome, but why should the pink be there at all? Now one of the effects of Sacculina on its host is to prevent it moulting, after the Sacculina has once come to the exterior. Consequently the infected crabs are not able to get rid of the red lipochrome at the moult in the ordinary way, and we might expect that an accumulation of this substance would take place in the blood.

Turning to the effect of Sacculina on Carcinus, we find on the contrary that the blood of infected individuals is invariably either colourless or else slightly yellow. We never get any perceptible development of the pink pigment,

and only very occasionally a moderately intense yellow coloration. It is very tempting to correlate this feeble formation of lipochrome in the blood of infected Carcinus with the very slight modification which the infected individuals undergo in their primary and secondary sexual characters in general, in comparison with Inachus. As Potts has shown, and I have repeatedly confirmed, the only effect of the parasite on Carcinus is to cause the abdomen of the male to become slightly broader in a certain percentage of cases; we never obtain the development of female appendages by the male or the full assumption of the female shape of the abdomen, such as frequently occurs in Inachus. The effect on the internal generative glands is also not nearly so well marked. The difference that Robson and I have found in the blood of the two infected forms, therefore, corresponds very well with the other features of the modification induced by the parasite in the two cases.

In the case of the effect of the parasite on the liver, however, our results agree completely. In both Inachus and Carcinus the infected crabs of both sexes almost invariably possess an opaque white or yellow liver in which a very large quantity of fat can be detected either by micro-chemical means or else by extraction methods. I have found in Carcinus that the liver of infected crabs is not only constantly full of fat but also deeply stained with yellow lipochrome, and this is the case at all times of year and in every phase of the infected animal's life, while the normal individuals exhibit great variations at different periods in the fat content of their livers. This, in itself, is an observation of first-rate importance in the theoretical interpretation of the effect of Sacculina on its host, since it proves beyond cavil that at any rate Sacculina does influence the fat metabolism of its host in a very positive degree. It is interesting to note also that in the effect on the liver Carcinus and Inachus react in the same way to the presence of the parasite; and this may be termed the first reaction to the parasite. The second reaction, namely the flooding of the

blood by an excess of fatty substance, only occurs to a considerable extent in the case of Inachus, and accordingly we find that this crab is influenced in every respect far more profoundly by the presence of the parasite than is the case in Carcinus.

To this point in the analysis of the effect on Sacculina on its host we have at present arrived, and though it is impossible to claim that the explanation is complete, yet we would crave the reader's indulgence while we remind him of the steps that have been won in advance of the position gained by Giard when he discovered the phenomenon of "parasitic castration." At that time the interpretation universally put upon the phenomenon was that the parasite caused the atrophy of the internal reproductive organs, and in consequence the secondary sexual characters of both sexes underwent correlative alterations, the male being assimilated to the female, and the female to the male.

The first step in advance of this interpretation was gained by showing that the alteration was always in the female direction, the female individuals never assuming any male characters under the influence of the parasite, whereas the male is frequently so highly modified towards the adult female state, both externally and internally, as to be easily mistaken for a true female. The next step was to show that not only was the male altered into the female condition, but also the young females themselves were forced under the influence of the parasite to assume prematurely the adult female characters.

The whole reaction, therefore, so far from being a passive result of the destruction of the gonad, was found to be an active assumption in both sexes of adult female characters, despite the entire absence of a functional ovary. A further step was gained by seeking in the internal condition of the animal's metabolism for the cause of these changes. It was clear that the old and familiar idea of an internal secretion produced by the gonad being the stimulus for the development of the secondary sexual

characters could not be applied here, since at the time that the alterations in the secondary sexual characters take place no ovary is present to give rise to the required stimulus. It was suggested, therefore, that in some way the stimulus must reside in the roots of the Sacculina, and it was observed that these roots were in process of elaborating from the blood of the host a pigmented yolk material, similar in every respect to the yolk which is stored in the ovary of a normal female crab at maturity. This clue formed the basis of the idea that the Sacculina roots, by continually abstracting the yolk material from the blood of the host, stimulated its constant production in excess, and that the presence of this material circulating in the body-fluids was the stimulus for the development of the female characters. An analogy was therefore drawn between this reaction and the reaction of warm-blooded animals to bacterial infections, and the phenomenon of parasitic castration was seen to approach the category of an immunity phenomenon. Mr. Robson and I have now attempted to follow more closely these internal changes in the fat-metabolism of the host, and a further step has been gained. We have shown that the blood of normal crabs does undergo periodic changes according to the sex of the animal, and that the blood of the female, at the time of the ripening of the eggs, is actually flooded by an excess of fatty material, which can be estimated quantitatively. Robson has found that in Inachus, where the parasitic affection is most complete, the blood of the infected individuals similarly is charged with fat material more constantly than is the case under normal conditions, while both in Carcinus and Inachus the infected individuals show a constant and excessive formation of fat in the liver.

It may therefore fairly be claimed that, though many points in our interpretation of the phenomenon may be lacking, yet on the whole the attempt to reduce the observed changes to an alteration in the condition of fat-metabolism has met with success, and that whereas formerly the

phenomenon of "parasitic castration" was merely an unaccountable curiosity, it now falls into place with other facts of female sexual organisation, and is to a great extent intelligible.

Summary.

(1) The blood of Carcinus mænas exhibits three chief conditions—colourless, pink, and yellow—the pink and yellow colour being due to the two lipochrome pigments, tetronerythrin and lutein. The pink colour appears in individuals, especially in males, which are approaching the period of a moult, the yellow is characteristic of the female when the ovary is approaching maturity.

(2) An estimation of the fat content of the blood, by means of saponification and extraction of the fatty acids, gives the following average numbers: for colourless blood ·059 per cent., for pink blood of males ·086 per cent., for yellow blood of females ·198 per cent. Thus the breeding females are shown to possess an excess of fatty material in the blood, and the yellow lipochrome is seen to represent a higher fat value than the pink.

(3) Beside the blood, the "liver" also exhibits periodic variations in the amount of fat present, fat being sometimes present as 12 per cent. of the total weight, sometimes as low as 4 per cent. The females maturing their ovary and having yellow blood always have a large proportion of fat in the liver.

(4) Crabs of both sexes infected with Sacculina also always show a large supply of fat in the "liver." In the case of Carcinus the blood of infected individuals is either colourless or pale yellow, but in the case of Inachus the blood becomes charged with lipochrome, as the result of infection, though this lipochrome always shows the presence of the pink colour as well as the yellow.

(5) The difference in reaction of the blood in the two cases is explained as consistent with the small effect which

Sacculina exerts on all the characters of Carcinus compared with its effects on Inachus.

(6) The investigation shows that Sacculina exerts a marked influence on the fat-metabolism of the host, and the results are on the whole consistent with the view that Sacculina influences the host to assume the female characters by acting the same part in the fat-metabolism as the ripening ovary does in a normal female.

Literature.

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